

# Regulation of Tension in the Skinned Crayfish Muscle Fiber

## II. *Role of Calcium*

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**ABSTRACT** Tension outputs were measured in skinned crayfish muscle fibers exposed to solutions variously buffered for both Mg-adenosine triphosphate (ATP) and Ca. Two types of data are shown, relating tension and substrate concentration with different levels of Ca present, or tension and calcium concentration at different levels of substrate. The data are fitted by curves calculated from a general equation for substrate inhibition. The equation is based on the schema that both tension and relaxation are induced by the substrate and that the relaxing effect of excess substrate is repressed by calcium. The physiological findings of the present work are similar to data obtained by others on biochemical model systems of the contractile proteins.

### INTRODUCTION

We have shown in an earlier paper (Reuben et al., 1971) that the skinned crayfish muscle fiber develops tension in the virtual absence of Ca ( $pCa > 9$ ) as the concentration of Mg-adenosine triphosphate (ATP), which is the substrate, is increased up to about  $3 \mu M$  ( $pMgATP = 5.5$ ) and that the fiber relaxes again when MgATP is increased further. An equation for a simple substrate inhibition model predicts quite well the bell-shaped curve relating tension to  $pS$ .<sup>1</sup> The effect of Ca was described briefly (Fig. 7 of Reuben et al., 1971) and we concluded, in agreement with Levy and Ryan (1965) and Chaplain (1967), that Ca "disinhibits" the relaxation (inhibition) induced

<sup>1</sup> The following abbreviations are employed:  $pCa$ ,  $pMg$ , etc., signify the negative logarithm of the concentration of the given species;  $S$  is substrate, MgATP or MgITP;  $S_o$  is the optimum substrate concentration for peak tension at a specified  $pCa$ ;  $P$  is tension and  $P_o$  the maximum tension developed by the skinned fiber;  $P'$  is the tension of the fiber before skinning when it is depolarized with 200 mM K. EDTA and EGTA are the chelating agents, ethylenediaminetetraacetic acid and ethylene glycol bis( $\beta$ -aminoethyl ether)  $N, N, N', N'$ -tetraacetic acid. A is actin, M is myosin, and AM is the complex actomyosin. SR is sarcoplasmic reticulum; KP is potassium propionate.

by excess substrate. The data on the effect of Ca are developed in this paper in two ways by studying: (a) the effect of Ca on the relation tension vs.  $pS$ , and (b) the relation for tension vs.  $pCa$  at different values of  $pS$ . These data are described by curves calculated from an equation that represents the general case of substrate inhibition with Ca present in specified concentrations.

#### METHODS

The skinned fiber was prepared, mounted in a flow chamber, and the tensions were recorded as described by Reuben et al. (1971). As in that work the fibers were skinned over 90% of their length. The fibers were about 5 mm long and, when depolarized by 200 mM K propionate (KP) before skinning, they produced tensions in excess of 1 g. The diameters and sarcomere lengths varied considerably. In one series of 24 fibers the diameters ranged between 138 and 360  $\mu$  ( $252 \pm 65 \mu$  sd) and the sarcomere lengths in the skinned preparation ranged between 6.9 and 11.3  $\mu$  ( $8.4 \pm 1 \mu$ ). The mean value of the sarcomere length is also that for maximum tension in crayfish muscle fibers (April, 1969).

The skinned fibers were exposed, at a constant temperature, to a series of solutions, of fixed  $pCa$  but of sequentially decreasing  $pS$ , which were recirculated continuously past the fiber. The solutions were made up to buffer simultaneously the  $pMg$ ,  $pCa$ , and  $pS$ . Usually the substrate ( $S$ ) was MgATP but Mg-inosine triphosphate (ITP) was used in some experiments. The methods and assumptions used to devise these solutions are given in the Appendix, and here we shall only describe the characteristics of the final solutions. All of the solutions contained 5 mM tris(hydroxymethyl) amino methane (Tris) maleate as a buffer and were titrated just before use to pH  $7.0 \pm 0.01$ . They usually contained 3 mM free ethylenediaminetetraacetate (EDTA) or ethylene glycol bis( $\beta$ -aminoethyl ether) $N,N,N',N'$ -tetraacetic acid (EGTA) total and 5 mM ATP, part of which was complexed with Mg. Any deviations from this pattern are given in the text. All the solutions were kept at an ionic strength of 0.25 by removal of KP to compensate for the concentrations of CaEDTA (or CaEGTA) and MgEDTA added to achieve the desired  $pCa$  and  $pS$ .

Besides the differences in the solutions, the only new methodological factor in the present work was the introduction of stricter temperature regulation. Just before the bathing solution entered the chamber it was passed through a heat exchanger maintained at the desired temperature, circulated past the fiber at a rate of several milliliters per second, and returned to the heat exchanger for recirculation. In most of the experiments cited the temperature was 20°C. Some experiments done at a lower temperature (11°C) will be described only briefly at this time.

#### RESULTS

Addition of Ca to solutions that contain MgATP modifies the bell-shaped relation between MgATP and tension of the skinned crayfish muscle fibers with  $pCa > 9$  that was described by Reuben et al. (1971). The results of three experiments on a single fiber are shown in Fig. 1. At  $pCa = 8$  the curve

is similar in shape, position, and relative tension output to the data previously obtained at  $pCa > 9$ . However, when  $pCa$  is reduced further the curves are different. The slope of the rising phase is the same, but relaxation occurs at lower values of  $pS$ . Thus the peak tensions are larger and the optimal substrate concentrations ( $pS_o$ ) are shifted to the right on the  $pS$  axis.

Similar data were obtained in all of the experiments and they are summarized in Table I. Part A shows the mean peak tensions ( $P/P'$ ) relative to the tension ( $P'$ ) evoked by 200 mM KP in the same fiber before skinning. At  $pCa > 8$  the ratio was  $28.2 \pm 8.3\%$  (number of experiments,  $N = 29$ ). It rose to  $42 \pm 9.2\%$  ( $N = 28$ ) with  $pCa$  in the range 7–6 and to  $59 \pm 5\%$  ( $N = 7$ ) with  $pCa < 6$ . The latter value is the maximum tension of the skinned fiber and is twice the value obtained in the virtual absence of Ca

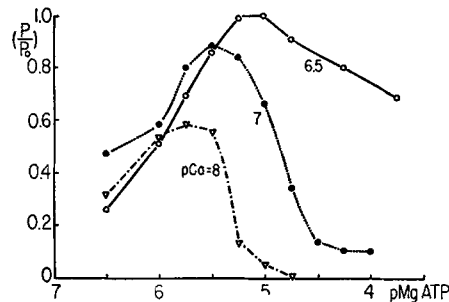


FIGURE 1. Tension as a function of  $pS$  in one fiber at three levels of  $pCa$  (8, 7, and 6.5, respectively). The tensions are normalized with respect to the peak tension at  $pCa = 6.5$ . The solutions were buffered with EDTA. Temperature  $20^{\circ}C$ .

(Reuben et al., 1971). Table I B list the mean values for the optimal substrate concentrations ( $pS_o$ ) for three different values of  $pCa$ : 8, 7, and 6.5, respectively, at  $20^{\circ}C$ .

The possibility that the buffering of Ca might be inadequate in the core of the fiber, because of the uptake of Ca by the sarcoplasmic reticulum (SR), was tested by including 10 mM caffeine in the solutions. This concentration is sufficient to depress uptake of Ca by SR (Weber and Herz, 1968). The presence of caffeine did not affect the experimental curves. This finding also rules out the possibility that caffeine might act *in vivo* by affecting in some way the association of the substrate and/or Ca with the contractile proteins.

Most of the data of the present work were obtained in solutions containing 5 mM total ATP. However, similar experiments were also done with a wide range of concentrations of ATP. No significant shifts in  $pS_o$  were observed until the ATP level was reduced below 1 mM with  $pCa < 6.5$  and below 0.2 mM with  $pCa \leq 8$ . At levels of ATP below these limits the curves failed to be bell shaped and the apparent optima were shifted to the right on the  $pS$

TABLE I

## A. Maximum tensions in different pCa

pCa	Relative tension ( $P/P'$ )		Number
	mean (%)	$\pm$ SD	
>8	28.2	8.3	29
6-7	42	9.2	28
<6	59	5.0	7

## B. Optimum substrate concentrations in different pCa

	pCa	$pS_o$ mean	$\pm$ SD	Number
At 20°C	8	5.7	0.18	12
	7	5.3	0.17	7
	6.5	4.88	0.12	9
At 11°C	6.5	5.75	—	3

$P'$  = tension of fiber with membrane intact on exposure to 200 mM K propionate.

$P$  = maximal tension of skinned fiber at designated pCa.

$P_o$  = tension when pCa < 6 and  $pS < 3$ .

$pS_o$  = substrate concentration for maximal tension in designated pCa.

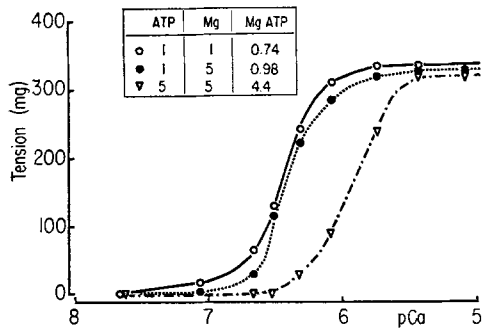


FIGURE 2

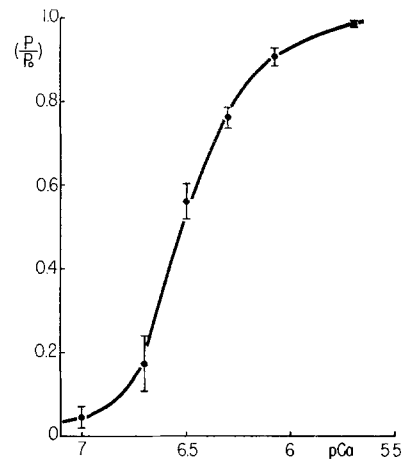


FIGURE 3

FIGURE 2. Tension as a function of pCa in one fiber at three levels of MgATP: 0.74, 0.98, and 4.4 mM, respectively. Solutions contained 3.5 mM EGTA (total).

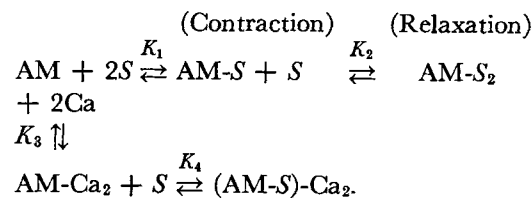
FIGURE 3. Composite curve of data on tension vs. pCa obtained in the presence of 3.3 mM EGTA (total) and 1 mM MgATP. Tensions were normalized as described in the text. Table II lists the averaged numerical data.

axis. This suggests that hydrolysis of the low concentrations of ATP reduces the pS of the solutions within the fiber from their calculated values. When the ATP in the solution is larger than the specified limits the supply of MgATP is buffered by the excess of ATP.

Complementary data relating the tension as a function of pCa for fibers initially relaxed in the presence of MgATP are shown in Fig. 2 which represents three experiments done on one fiber. The solutions contained 3.5 mM EGTA but Mg and ATP were present in different proportions. The calculated concentrations of MgATP were 0.74, 0.98, and 4.4 mM, respectively. An increase in MgATP shifted the tension vs. pCa curves to the right on the pCa axis, but the curves are essentially similar, sigmoid in form, with their rising phase extending over about one pCa unit. The maximum tensions ranged between 320 and 340 mg, and at pCa < 5.5 were essentially independent of the concentration of substrate.

Similar data were obtained in other experiments of this type and Fig. 3 shows the measurements collected in the presence of 3.3 mM EGTA and 1 mM Mg and ATP, respectively. The tensions were normalized with respect to those obtained at the lowest value of pCa ( $\cong 5.3$ ). As seen in Fig. 2, the maximum tension ( $P_o$ ) that the skinned fibers can produce develops at pCa  $\leq 5.5$ . The numerical values are shown in Table II.

The data of Figs. 1-3 and Tables I and II provide a test of the hypothesis that both tension and relaxation are effected by MgATP as the substrate which induces substrate inhibition of the actomyosin (AM)-ATPase according to the schema proposed by Reuben et al. (1971). For the purpose of the present treatment the schema is now generalized to include the effects of Ca as follows



The equilibrium equation for this schema is:

$$P = \frac{P_o}{1 + \frac{K_1}{S} + \frac{S}{K_2(1 + \text{Ca}^2/K_3)}} \quad (1)$$

where it is assumed that

$K_1 \cong K_4$ , where  $K_1$ - $K_4$  are dissociation constants,

$P$  is the tension, proportional to  $AM \cdot S + (AM \cdot S) Ca_2$ , and  $P_o$  is the maximum tension of the skinned fiber and is proportional to the sum of all forms of AM present. In resting muscle AM is mostly dissociated. However,  $AM \cdot S_2$  is probably a precursor of this dissociation process.

Differentiating equation 1 with respect to substrate and setting  $dP/dS = 0$  yields the relation between optimal substrate concentration ( $S_o$ ) for any given pCa, as well as the three dissociation constants ( $K_1 - K_3$ ). Then,

$$S_o^2 = K_1 K_2 (1 + Ca^2 / K_3) \quad (2)$$

or, in a form suitable for linear regression analysis,

$$Ca^2 = \frac{S_o^2 K_3}{K_1 K_2} - K_3. \quad (3)$$

The schema and equation are written assuming that two Ca ions participate in lifting the substrate inhibition. Two types of data appear to indicate this requirement. In the first type the mean  $S_o$  values (Table I B) at each pCa were tested in a linear regression program against the Ca concentration, raised to the first power, squared, or cubed, respectively. The

TABLE II  
DEPENDENCE OF TENSION ON pCa  
(DATA OF FIG. 3)

pCa	$P/P_o$	SE	$N$
7.0	0.046	$\pm 0.024$	21
6.7	0.176	0.06	21
6.5	0.56	0.04	25
6.3	0.76	0.03	26
6.08	0.91	0.02	24
5.7	0.99	0.003	39

regression coefficients were 0.985 for the first, 0.9996 for the second, and 0.996 for the third power, respectively, and the second power was adopted. The regression analysis also provided estimates for the dissociation constants  $K_1 - K_3$ . The value  $K_1 \cdot K_2$  was identical with that obtained in the earlier work (Reuben et al., 1970), when the best fit for the bell-shaped curve with pCa > 9 was obtained with  $K_1 = 1.5 \mu M$  and  $K_2 = 4 \mu M$ . Hence,  $K_2$  was evaluated as  $0.0036 \mu M^2$ . It can be converted to a binding constant for Ca in the first power by taking the reciprocal of the square root. The value so obtained ( $1.5 \times 10^7 M^{-1}$ ) is identical with the binding constant obtained for the "higher" affinity site by Weber (1970). The binding constant can also be obtained directly by regressing to the data of Table I B without squaring the Ca term. This yields a value of  $2-4 \times 10^7 M^{-1}$ .

The second type of evidence is the fit of the relation shown in Figs. 2 and 3 by curves calculated from equation 1. Such curves were calculated on the basis of the first and second power of Ca in the equation. With Ca set at the first power the sigmoid curve extended over at least two pCa units. Entering Ca as the square in equation 1 gave curves very similar to those of Figs. 2 and 3, extending over about one pCa unit. The curves of Fig. 4 were calculated in this manner for five different concentrations of the substrate. They predict very well the data of Fig. 1 and of the measurements recorded in Fig. 7 of the previous paper (Reuben et al., 1971). The tension calculated for  $pS = 6$  (Fig. 4) is somewhat less than the peak tension at the optimum ( $pS = 5.5$ ). This remains essentially unchanged as pCa is varied over a wide range, in keeping with the experimental finding that at low substrate concentrations the tension on the rising phase is not altered by increasing Ca. Increasing substrate to  $pS = 5$  reduces the calculated initial tension, indicating that the latter is below its peak value and is on the falling phase of the

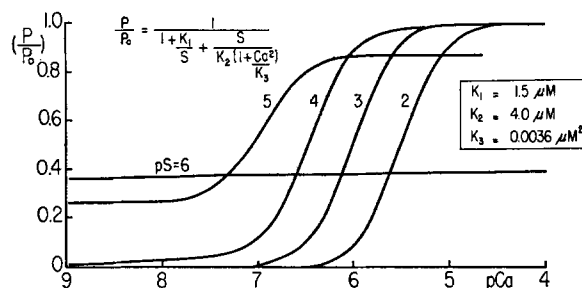


FIGURE 4. Curves relating normalized tension ( $P/P_0$ ) and pCa for five levels of pS calculated from equation 1 of the text.  $K_1 - K_3$  were obtained from data with EDTA-buffered solutions.

tension vs. pS curve with  $pCa > 8$ . The calculated tension now increases along a sigmoid curve as Ca is increased. With further increase of the substrate concentration by decade steps the calculated tension is zero at  $pCa > 8$  but increases to a maximum value as pCa is reduced. The sigmoid curves are displaced to the right on the pCa axis as pS is reduced.

Equation 1 was also used to calculate the relation between tension and pS for different values of pCa (Fig. 5). For  $pCa = 8$  the bell-shaped curve is identical with that calculated in the previous paper (Fig. 6 of Reuben et al., 1971). The curves obtained with  $pCa = 7$  and  $6.5$ , respectively, fit the experimental data well (e.g. Fig. 1) although the fit is not necessarily perfect for any given set of experimental data. The peak tension at  $pCa = 8$  is predicted to be half the maximum tension in saturating concentrations of Ca in agreement with the experimental results (Table I A).

A few experiments were done at low temperature (Table I B), where it was noted that the curves of tension vs.  $pS$  were shifted to the left. At the same time the amplitudes of the tensions decreased. With  $pCa \leq 8$  the fibers at  $11^\circ C$  were completely relaxed in the lowest concentrations of substrate that could be obtained in the presence of 3 mM EDTA and, thus, no value for  $pS_0$  could be obtained. However, with  $pCa = 6.5$ ,  $pS_0$  was 5.75 or nearly an order of magnitude greater than at  $20^\circ C$ . As  $pS$  increased in the cooled fibers the maximum tension decreased. A similar shift with temperature was also observed by Levy and Ryan (1967) in measurements of syneresis. We conclude, therefore, that while both  $K_1$  and  $K_2$  may be sensitive to temperature,  $K_2$  decreases most with decrease in temperature. These findings re-enforce the need, stated in the earlier paper (Reuben et al., 1971) for strict temperature stabilization of the experimental regime.

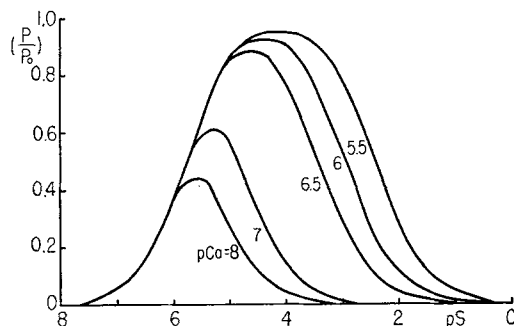


FIGURE 5. Curves relating normalized tension ( $P/P_0$ ) and  $pS$  for five levels of  $pCa$  calculated from equation 1 of text.

Some experiments were done with MgITP as the substrate. Although the values for  $pS_0$  were about three units lower with MgITP than with MgATP (Reuben et al., 1971) the curves of tension vs.  $pS$  shifted with  $pCa$  to about the same degree as with MgATP. This finding indicates that the substitution of ITP for ATP affects the dissociation constants  $K_1$  and  $K_2$  but does not alter  $K_3$ . Ebashi et al. (1968) found that the binding of Ca to troponin is not affected by the concentration of ATP.

#### DISCUSSION

Studies on the biochemistry of the contractile proteins suggested to a number of workers that substrate inhibition is involved in the dissociation of the contractile proteins and, by inference, in relaxation of muscle (see Stewart and Levy, 1970; Reuben et al., 1971, for references). The data of this and of the previous paper (Reuben et al., 1971) are in agreement with the view of Levy and his colleagues (Levy and Ryan, 1965, 1967; Stewart and Levy, 1970) and Chaplain (1967) that the role of Ca is to repress or suppress the inhibi-



tory interaction between excess MgATP and the contractile proteins. MgATP is present in high concentration in vivo, while the concentration of free Ca is low, so that the muscle is relaxed until there is a release of Ca (see Sandow, 1965, 1970; Weber, 1966; Ebashi and Endo, 1968; Bendall, 1969).

The general equation of substrate inhibition which includes the role of Ca permits calculation of the theoretical relations expected for tension vs. pS and vs. pCa, respectively, for a series of fixed values of pCa or pS. The good agreement between the data (Figs. 1-3, Table I) and the predictions (Figs. 4 and 5) indicate that the substrate inhibition model is sufficient to account for the physiological data on the tension of skinned muscle fibers.

#### *Comparison with Other Data*

A survey of some of the very extensive literature indicates that the substrate inhibition schema generalized to include the effect of Ca predicts a wide variety of data on model actomyosin systems tested in the presence of calcium. The two general predictions summarized in Figs. 4 and 5 are as follows.

- (a) At a fixed and appropriate pS the manifestation of "active state" in the contractile proteins increases with increasing concentrations of Ca.
- (b) At a fixed pCa the active state increases to an optimum, as substrate is increased, then decreases.

The correspondence between the different indexes of active state applied in muscle preparations which range from skinned fibers to actomyosin gels may be incomplete. Superprecipitation of actomyosin gels or myofibril preparations seems to be the analogue of tension in skinned fibers and we assume that it is a comparable index of the active state. For example, the bell-shaped curve obtained for syneresis in the absence of Ca peaks at about half the maximum degree estimated when Ca is present (estimated from the shape of the curve 18.1 B, in Weber, 1970) in parallel with the tension of the skinned muscle fiber. However, the bell-shaped curve obtained for ATPase rates in the absence of Ca is abolished by Ca addition and the hydrolysis continues to increase progressively with substrate concentration well beyond double the peak obtained in the absence of Ca (Weber, 1969, and Fig. 18.1 A in Weber, 1970).

#### *Role of Ca*

Weber and Winicur (1961) and Weber et al. (1964), using Ca EGTA buffers, obtained sigmoid curves for the relation between pCa and ATP hydrolysis by actomyosin preparations. These began to rise at a "threshold" near pCa = 7, and were steep, the bulk of the curve occupying about one pCa unit (between 7.5 and 6.5). Because the curve was so steep they suggested that two Ca ions are required per cross-bridge (active site) for full activation of the AM system. They also found that Ca binding and syneresis increased

nearly in parallel with the ATPase activity. Filo et al. (1965) demonstrated this sigmoid relation in glycerinated psoas muscle where the tension increased over the range  $pCa = 7-6$ . Hellam and Podolsky (1969) obtained a sigmoid relation for frog muscle fibers skinned by the Natori (1954) method. The data summarized above are quite comparable to and show good agreement with the data of our Figs. 2 and 3 and the predictions of Fig. 5.

Ebashi and Endo (1968) reported a rather different range for the  $pCa$  sensitivity of their skinned frog fiber preparations. Most of the discrepancy appears to be due to their use of a much lower apparent association constant for CaEGTA. That value would have caused a shift of the curve of Fig. 3 to the right by nearly one  $pCa$  unit. There is, in fact, a discrepancy in our data between curves obtained with solutions buffered for Ca with EDTA and those buffered with EGTA. This may be seen by comparing the curve of Fig. 3, representing data obtained in CaEGTA buffers, with the curve at  $pS = 3$  of Fig. 4, calculated for EDTA-buffered solutions.

The rising phases of the curves of Figs. 1 and 5 are determined by  $K_1$  of equation 1. The slope of the rising phase is the same in the presence of high Ca as when Ca is virtually absent. Chaplain (1967) shows a similar effect on ATPase rates of insect actomyosin. The rate of superprecipitation of AM gels at low substrate concentrations is about the same whether Ca is present or not (Stewart and Levy, 1970) in accord with the data of Fig. 7 of our previous paper (Reuben et al., 1971) and with the absence of an effect of Ca on the slope of the rising phase in the curves of Figs. 1, 4, and 5. Weber (1970, Fig. 18.2) also found that ATPase activity changed very little with increasing Ca concentration when the substrate concentration was low (5 mM Mg and 0.01 mM ATP). Hence it may be concluded that binding of Ca to troponin-tropomyosin (Ebashi et al., 1968) does not to any significant degree affect the binding of substrate to the excitatory site of actomyosin.

#### *Substrate Inhibition in the Presence of Calcium*

In contrast to the numerous studies on the effect of different concentrations of Ca on the activity of various muscle preparations, there are only a few studies on the effects of different substrate concentrations at fixed  $pCa$ . Filo et al. (1965), studying glycerinated muscle, increased the substrate concentration in a systematic way at a fixed  $pCa$  through part of the critical range represented by our Fig. 5. An optimum was observed at low concentrations of Ca and at a higher concentration of Ca there was both a shift of the optimum and a larger tension. However, the full curve at a high concentration of Ca was not generated in their study. Ebashi and Endo (1968) related  $pCa$  to rates of superprecipitation in media containing 1 mM ATP and

1, 4, and 8 mM Mg. The curves shifted progressively to the right and at least in part this may be because the concentration of MgATP was increasing from about 0.7 to 1.0 mM. Levy and Ryan (1967), Chaplain (1967), and Dancker (1970) also reported data which suggest a shift in the optimal substrate concentration with Ca and, in our earlier paper (Reuben et al., 1971, Fig. 7), one experiment of such an effect on tension was included. All of the foregoing data indicate that maximum effects can be obtained in  $pS = 3$  or  $2$  when Ca is present in saturating concentrations, whereas in the absence of Ca approximately half the maximum tension occurs at an optimum  $pS_0 = 5.5$  (Reuben et al., 1971).

Thus the quantitative antagonism between substrate concentration and Ca concentration appears to be established for a variety of measures of the active state of actomyosin systems. A direct biochemical measure of the antagonism between substrate and Ca is suggested in Fig. 18.3 of Weber (1970), which shows that myofibrils bind less MgATP in the presence of Ca than in its absence. Therefore the substrate inhibition schema embodied in equation 1 provides a quantitative basis for testing various models of the molecular biology of contraction.

#### APPENDIX

A programmed calculator (Wang Laboratories, Inc., Tewksbury, Mass., 370-2) was used to provide mixing instructions for each EDTA-buffered solution, from stock solutions containing, respectively, 66 mM  $K_2$  EDTA, 66 mM CaEDTA, 66 mM MgEDTA, and 200 mM KP. All of these stock solutions contained 5 mM Tris-maleate buffer. The calculator was programmed to determine the final concentrations of the ingredients and then to calculate the necessary volumes of each stock solution to be mixed so as to obtain the desired solution.

Four conditions were specified initially:  $pS$ ,  $pCa$ , and the concentrations of free EDTA and ATP total. From these specifications the following equations can be solved sequentially:

$$\frac{(\text{CaEDTA})}{(\text{Ca})(\text{EDTA})} = K_{a(\text{CaEDTA})} \quad (1a)$$

$$\text{ATP}_{\text{Total}} = \text{ATP}_{(\text{free})} + \text{MgATP} \quad (2a)$$

$$\frac{(\text{MgATP})}{(\text{Mg})(\text{ATP})} = K_{a(\text{MgATP})} \quad (3a)$$

$$\frac{(\text{MgEDTA})}{(\text{Mg})(\text{EDTA})} = K_{a(\text{MgEDTA})} \quad (4a)$$

$$\text{EDTA}_{\text{Total}} = \text{MgEDTA} + \text{CaEDTA} + \text{EDTA}_{(\text{free})}. \quad (5a)$$

Equation 1 *a* has only one unknown (CaEDTA). Combining equations 2 *a* and 3 *a* leaves only one unknown (Mg). Together Equations 1 *a*–3 *a* specify equation 4 *a* so that only one unknown (MgEDTA) remains. It should be noted that one equation, that for the reaction between Ca and free ATP

$$\frac{(\text{CaATP})}{(\text{Ca})(\text{ATP})} = K_{a(\text{CaATP})}, \quad (6a)$$

is omitted. For the range of concentrations of Mg ATP and free ATP used this reaction perturbs the concentrations calculated as above by < 1%. At pCa = 5, which is below the lowest pCa used, CaATP is only 5% of the free ATP calculated from equation 2 *a*. In most of the solutions used CaATP was several orders of magnitude smaller.

To prepare a series of substrate concentrations at a fixed pCa, equation 1 was first solved and the 66 mM CaEDTA stock was mixed with each of the other stock solutions so that they all contained the same concentration of CaEDTA. The procedure diluted these other stocks and that information was fed into the program at the point where it determined the volumes of these stocks to be mixed into the final bathing solution. ATP was added to the final mixture from a stock solution of 200 mM ATP titrated to pH 7.0 with KOH, stored in the freezer until use, and kept on ice when out of the freezer.

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